SHORT COMMUNICATION

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Allelic differences in *Medicago truncatula NIP/LATD* mutants correlate with their encoded proteins' transport activities in planta

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Abbreviations: MtNIP/LATD, *M. truncatula* numerous infections and polyphenolics/lateral root-organ defective; N, nitrogen; NAR2, nitrate assimilation related 2; NRT1(PTR), nitrate transporter 1(peptide transporter); CHL1, chlorate 1; NRT2, nitrate transporter 2

Medicago truncatula NIP/LATD gene, required for symbiotic nitrogen fixing nodule and root architecture development, encodes a member of the NRT1(PTR) family that demonstrates high-affinity nitrate transport in *Xenopus laevis* oocytes. Of three Mtnip/latd mutant proteins, one retains high-affinity nitrate transport in oocytes, while the other two are nitrate-transport defective. To further examine the mutant proteins' transport properties, the missense *Mtnip/latd* alleles were expressed in *Arabidopsis thaliana chl1-5*, resistant to the herbicide chlorate because of a deletion spanning the nitrate transporter *AtNRT1.1(CHL1)* gene. *Mtnip-3* expression restored chlorate sensitivity in the *Atchl1-5* mutant, similar to wild type *MtNIP/LATD*, while *Mtnip-1* expression did not. The high-affinity nitrate transporter *AtNRT2.1* gene was expressed in *Mtnip-1* mutant roots; it did not complement, which could be caused by several factors. Together, these findings support the hypothesis that MtNIP/LATD may have another biochemical activity.

Most legumes are able to thrive in nitrogen (N) depleted soils because they can assimilate N via N fixing root nodules, symbiotic organs formed in partnership with rhizobia. Nodulation begins with an exchange of signals between the symbiotic partners, followed by plant cell divisions in the root cortex and pericycle and subsequent rhizobial invasion of the root through plant-derived infection threads. The infection threads in newly divided cells are encased by thin cell walls that can breach, forming an infection droplet. At the site of droplet formation, the plasma membrane separating the bacteria from the plant cytoplasm continues to proliferate, and individual rhizobia are endocytosed into the plant host cell cytoplasm, forming symbiosomes. Within effective nodules, bacteria establish a long-term infection within plant tissues, and additional differentiation of both plant and bacterial components occurs before N fixation commences. For more details, consult recent reviews.[1-3]

In *Medicago truncatula*, the *NIP/LATD* gene is essential for establishment of an effective N fixing symbiosis.[4-9] However, *MtNIP/LATD* is not required for the initial stages of rhizobial invasion into host roots, suggesting a role in a plant checkpoint that occurs between bacterial invasion and the establishment of an intracellular infection. Three *M. truncatula* mutants defective in *NIP/LATD* have been studied. The *Mtnip-3* mutant has mild defects in lateral root (LR) elongation and develops symbiotic nodules with significantly reduced N fixation,[7] whereas the *Mtnip-1* mutant exhibits severe LR defects and defective nodules with rhizobia in infection threads but only rare rhizobial release into symbiosomes.[4,5] The *Mtlatd* mutant has the most severe phenotype of the three alleles, with stunted root architecture and nodules similar to those found in *Mtnip-1*.[6]

MtNIP/LATD encodes a protein in the NRT1(PTR) family, suggesting that MtNIP/LATD functions in small molecule transport. Members of the NRT1(PTR) family transport di- and tri-peptides, hormones, glucosinolates and other compounds.[10-14] Many NRT1(PTR) transporters are low affinity nitrate transporters.[10] High affinity nitrate transporters are mostly found in the evolutionarily distinct NRT2 transporter family,[10] although two dual affinity nitrate transporters in the NRT1(PTR) family have been described, *Arabidopsis thaliana* NRT1.1(CHL)[15] and *M. truncatula* NRT1.3.[16] MtNIP/LATD protein was found to transport nitrate with a Km of 160 μM in *Xenopus laevis* oocytes, indicating that it is a high-affinity nitrate transporter.[17] *Mtnip-1*

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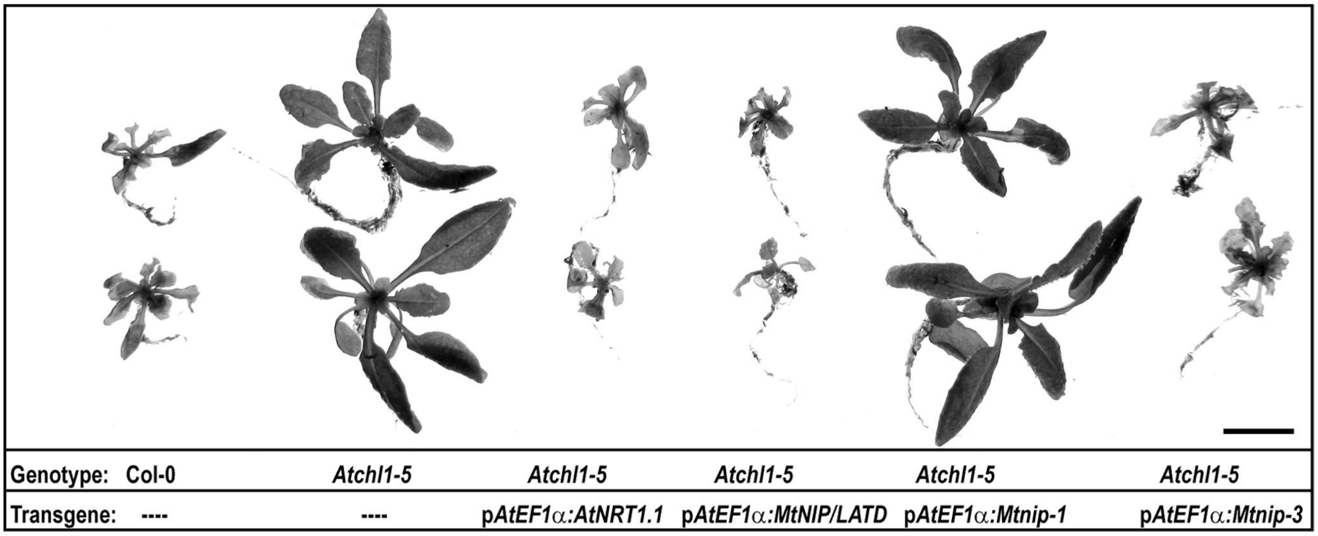

Figure 1. *Mtnip-3*, but not *Mtnip-1*, complements the chlorate-insensitivity phenotype of the *Arabidopsis chl1-5* mutant. *Arabidopsis* control and test plants were treated with chlorate, a nitrate analog that can be converted to toxic chlorite after uptake.[17,20] Two independent *Atchl1-5/Mtnip-1* lines and three independent *Atchl1-5/Mtnip-3* lines were tested; representative plants are shown. The genotype and transgene in each plant is indicated. Bar = 1 cm. The *Mtnip-3* gene was able to confer chlorate sensitivity on *Arabidopsis chl1-5* plants, similar to the *MtNIP/LATD* and *AtNRT1.1* genes.

and *Mtnip-3* have missense mutations, causing amino acid sequence changes A497V and E171K respectively, while *Mtlatd* has a nonsense mutation, W341Stop, in the *MtNIP/LATD* gene.[8] Mtnip-1 and Mtlatd proteins were found not to transport nitrate in oocytes, but Mtnip-3 transported nitrate indistinguishably from wild type.[17] This suggests that Mtnip-3 may be defective in transport of another compound, or could be defective in a different activity that is responsible for the phenotypes observed in the *Mtnip-3* mutant.[7,17] It is also possible that Mtnip-3 is capable of transporting nitrate in oocytes, but for some reason is not able to do so in planta. Here we further examine transport properties of the mutant proteins in planta. We also examine whether the phenotypes of a *Mtnip/latd* mutant can be rescued by expression of a high-affinity nitrate transporter.

To examine transport properties of the mutant *MtNIP/LATD* alleles in planta, we expressed the alleles separately in the *A. thaliana chl1-5* mutant, with a deletion spanning the *AtNRT1.1(CHL1)* gene,[18] encoding a major dual affinity nitrate transporter.[19] The *Atchl1-5* mutant was originally isolated on the basis of its resistance to chlorate, an herbicide and nitrate analog that is transported through the AtNRT1.1(CHL1) nitrate transporter[20] into roots where it is converted to phytotoxic chlorite. Therefore, the expression of functional nitrate transporters in *Atchl1-5* results in the loss of chlorate resistance and a reduction of plant vigor after chlorate treatment. The two mutant alleles encoding missense mutations in their *MtNIP/LATD* were investigated in *Atchl1-5* for their ability to restore sensitivity, and plant vigor was determined by documenting overall plant size, mass and chlorophyll content. Two *Atchl1-5* lines independently transformed with a constitutively expressed *Mtnip-1* and three independent *Atchl1-5* lines expressing *Mtnip-3* cDNA were selected for further analysis, based on similar, robust *Mtnip-1* or *Mtnip-3* mRNA expression compared with wild-type *MtNIP/LATD* expression in control *Atchl1-5* lines expressing wild-type *MtNIP/LATD*.[17]

Atchl1-5 plants expressing *Mtnip-1* or *Mtnip-3* and controls were treated with chlorate, as described previously.[17,20] *Mtnip-3* cDNA expression in *Atchl1-5* restored chlorate sensitivity (representative plants are in **Fig. 1**, far right; shown in color in **Fig. S1**) and resulted in a dramatic reduction in plant size, as did the expression of positive controls, *AtNRT1.1* cDNA and *MtNIP/LATD* cDNA (**Fig. 1**; **Fig. S1**). In contrast, *Mtnip-1* expressing *Atchl1-5* plants were found to be chlorate resistant and were indistinguishable from *Atchl1-5* plants (**Fig. 1**; **Fig. S1**). The vigor of *Atchl1-5* plants expressing test genes in trans was determined by measuring fresh weight (**Fig. 2A**) and chlorophyll content (**Fig. 2B**) after treatment with chlorate. The fresh weights of the three lines of *Atchl1-5* expressing *Mtnip-3* cDNA were indistinguishable (lines 1 and 2) or slightly larger than (line 3) the weights of *Atchl1-5* plants transformed with *AtNRT1.1* or *MtNIP/LATD* expression constructs (**Fig. 2A**). In contrast, the masses of plants from the two independent *Atchl1-5/Mtnip-1* lines were indistinguishable from those of negative control *Atchl1-5* plants, indicating that they retained chlorate resistance (**Fig. 2A**). Chlorophyll content was also measured in extracts from chlorate-treated plants expressing the control or test genes. The chlorophyll content of *Atchl1-5/Mtnip-3* lines 1 and 2 were similar to those of the *Atchl1-5* lines expressing the positive control *AtNRT1.1* or wild-type *MtNIP/LATD* and to wild type Col-0, while the third line, *Atchl1-5* line 3, had a slightly higher chlorophyll content than the positive controls (**Fig. 2B**). Both *Atchl1-5/Mtnip-1* plants had higher chlorophyll content than *Atchl1-5* plants expressing *AtNRT1.1*, *MtNIP/LATD* or *Mtnip-3*, but slightly lower than *Atchl1-5* control plants (**Fig. 2B**). Collectively, these data indicate that MtNIP/LATD and Mtnip-3 proteins transport chlorate in planta (**Figs.

1F and 2A), although transport by Mtnip-3 protein may be slightly less efficient than wild-type MtNIP/LATD (**Fig. 2B**). Similarly, the data indicate that Mtnip-1 protein is non-functional (**Figs. 1E and 2A**) or retains a very small fraction of transport activity (**Fig. 2B**).

Previously, we tested the expression of two *NRT1(PTR)* genes, the aforementioned *AtNRT1.1(CHL1)* gene and *AgDCAT*, encoding a dicarboxylate transporter,[13] for their abilities to rescue *Mtnip-1* phenotypes when expressed in *Mtnip-1* roots. We found that expression of *AtNRT1.1* in *Mtnip-1*'s roots partially rescued the root architecture phenotype,[17] supporting the hypothesis that MtNIP/LATD in *M. truncatula* transports nitrate at high affinity. However, because AtNRT1.1 also transports auxin[11] and acts as a nitrate sensor,[21] it cannot be ruled out that one of these other activities is responsible for the phenotype of *Mtnip-1* roots constitutively expressing *AtNRT1.1*.[17] *AgDCAT* expression in *Mtnip-1* was without effect on root or nodule phenotypes.[17] Because MtNIP/LATD was shown to transport nitrate with high affinity in oocytes, here we tested the *MtNIP/LATD* promoter-driven expression of *AtNRT2.1,* encoding a high-affinity nitrate transporter, in *Mtnip-1* roots to determine if it complemented the phenotypic defects. We found that *AtNRT2.1* expression had no effect on the *Mtnip-1* phenotypes, with neither root architecture nor nodulation defects altered (**Fig. S2**). While this might suggest that the important biological role of MtNIP/LATD is not in nitrate transport, non-complementation of *Mtnip-1*'s phenotype by *AtNRT2.1* may be due to other factors. One possible factor is the requirement for proteins in the AtNRT2 family to interact with proteins in the NAR2.1, also called NRT3, family for functional high-affinity nitrate transport.[22] Another is the apparent post-transcriptional control of AtNRT2.1.[23] Thus, this negative result may not be informative.

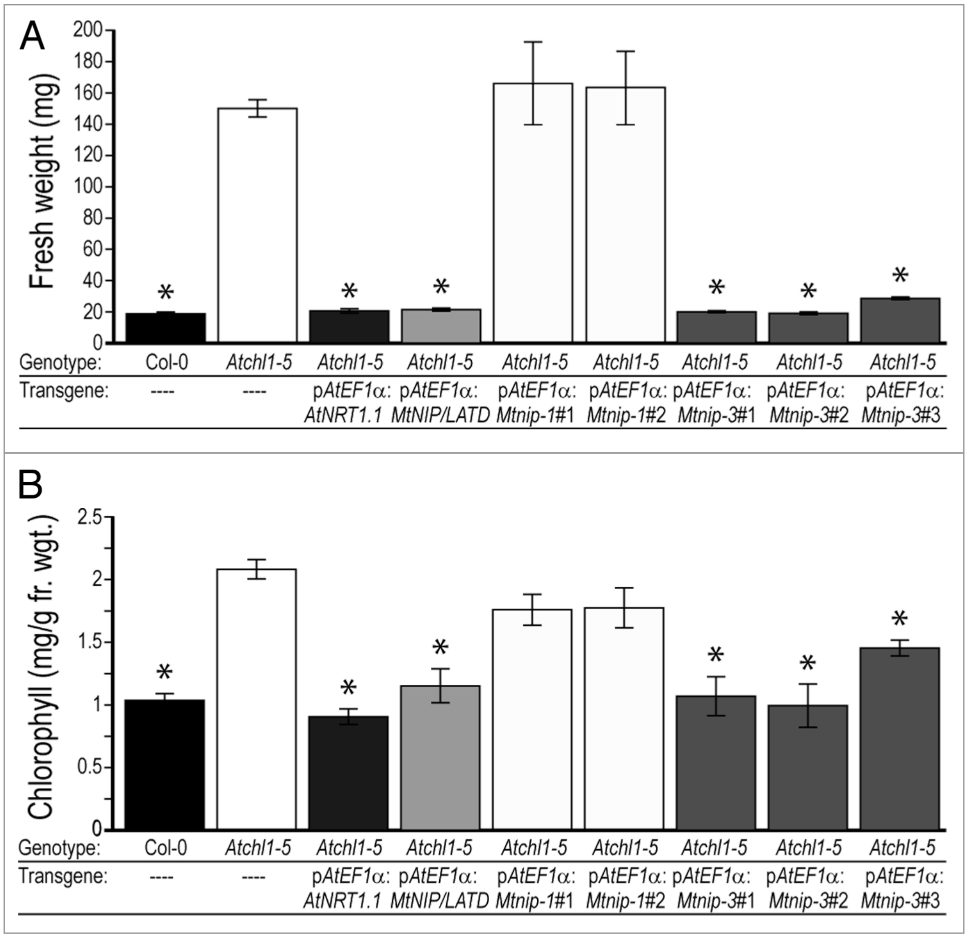

Figure 2. Fresh weight and chlorophyll content of chlorate treated *Arabidopsis* plants. The fresh weights and chlorophyll content of the *Arabidopsis* Col-0, *Atchl1-5 and Atchl1-5* plants transformed with constructs and treated with chlorate, as in **Figure 1**, were measured. Asterisks mark values that are significantly different at the 1% level from the *Atchl1-5* value, using the paired t-test. (**A**) The fresh weights of *Atchl1-5* plants transformed with the *Mtnip-1* gene were indistinguishable from *Atchl1-5* plants, and the fresh weights of two of the *Atchl1-5* lines transformed with *Mtnip-3* were indistinguishable from those of Col-0 or *Atchl1-5* transformed with either *MtNIP/LATD* and *AtNRT1.1* genes. The third *Atchl1-5/Mtnip-3* line has similar, but not identical, fresh weight to the other two. (**B**) The chlorophyll contents of *Atchl1-5* plants transformed with the *Mtnip-1* gene were similar to *Atchl1-5* plants, and the chlorophyll contents of two of the *Atchl1-5* lines transformed with *Mtnip-3* were similar to those of Col-0 and to chlorophyll contents of *Atchl1-5* transformed with either *MtNIP/LATD* and *AtNRT1.1* genes. The third *Atchl1-5/Mtnip-3* line had intermediate chlorophyll content.

In conclusion, previously we found that MtNIP/LATD and Mtnip-3 proteins transport nitrate in oocytes, while the Mtnip-1 and Mtlatd proteins did not. Here, we show that *Mtnip-3* expression, like that of *MtNIP/LATD,* complements the chlorate resistance of the *Arabidopsis chl1-5* mutant, while *Mtnip-1* does not. This new data demonstrates that Mtnip-3 transports the nitrate analog chlorate in planta, thus, strongly suggesting that Mtnip-3 protein is proficient at nitrate transport in planta. This result is consistent with the previous observation that Mtnip-3 transports nitrate in oocytes.[17] However, the *Mtnip-3* mutant has root architecture and nodulation defects in *M. truncatula*.[7,8] Together, these findings suggest that Mtnip-3 is defective in a second, so far unknown, biochemical activity found in MtNIP/LATD. Finally, we found that *AtNRT2.1* does not complement any of the phenotypes found in *Mtnip-1*. However, complementation of *Mtnip-1* by *AtNRT2.1* may require co-expression of *AtNAR2.1* [22] or post-transcriptional modulation,[23] and thus non-complementation by *AtNRT2.1* alone may not be informative.

Materials and Methods

***A. thaliana* plant constructs and chlorate tests.** *Mtnip-1* and *Mtnip-3* cDNA were amplified from oocyte expression vectors used previously[17] using primers NIPC2F (TGA ACC ATG GAG TAC ACA AAC AGT GAT GAT GCT AC) and NIPCODBst1R (AAA AAG GTC ACC TAT GAA GTA GGC AAC TCC CTG T) and subsequently cloned into NcoI and BstEII site of pMS004[17] to create pYM001 and pYM002. Then BamHI/BstEII fragments from pYM001 and pYM002 were cloned into pCAMBIA2301 to create pYM003 (pCAMBIA2301-p*AtEF1*α-*Mtnip-1*) and pYM004 (pCAMBIA2301-*AtEF1*α-*Mtnip-1*), yielding binary vectors with the mutant alleles under the control of the constitutive *AtEF1*α promoter. pYM003 and pYM004 were transformed into *Agrobacterium tumefaciens* GV3101(pMP90) cells and then transformed into *Atchl1-5* mutant by the floral dip method.[24] Homozygous plants for each line were selected on half strength MS medium (Research Products International) containing 25 mg/L kanamycin. RNA was extracted from each line (Qiagen, RNeasy kit), contaminating DNA removed (Life Technologies, Ambion TURBO DNA-free kit; then Qiagen RNeasy MinElute kit) and cDNA synthesized (Life Technologies, Invitrogen SuperScript First-Strand Synthesis system). The cDNA was subjected to semi-quantitative RT-PCR for levels of *MtNIP/LATD* or mutant allele mRNA expression using primers NipC2F (TGA ACA TGG AGT ACA CAA ACA GTG ATG ATG CTA C) and NipCSeq2R (TTC TTG GTT GCC ACC ACA AC). Independent lines having similar expression levels were selected for further analysis (data not shown). Chlorate sensitivity tests were performed as described.[17,25] *Mtlatd*, because it has a nonsense mutation in the middle of the *MtNIP/LATD* coding region,[8] was not tested for its ability to complement the *Atchl1-5* mutant.

***AtNRT2.1* expression in *M. truncatula*.** A 1.7 Kbp *AtNRT2.1* cDNA was amplified from an *AtNRT2.1* cDNA clone obtained from Dr. Anthony Glass using primers NRT2.1BamHIF (CTA GGG ATC CAT GGG TGA TTC TAC TGG TGA GCC G) and NRT2.1BstEIIR (ATG AGG TAA CCT CAA ACA TTG TTG GGT GTG TTC TCA GGC GG). Subsequently, the 1.7 Kbp *AtNRT2.1* cDNA was cloned into BglII and BstEII sites of pMS014, a vector containing the 3 kb *MtNIP/LATD* promoter with an EcoRI site at its 5' end and a BglII site at its 3' end, to create pMS016. Subsequently the EcoRI/BstEII fragment was cloned into pCAMBIA2301 creating pMS020 (pCAMBIA2301-p*MtNIP/LATD-AtNRT2.1*). pMS020 was transformed into *Agrobacterium rhizogenes* MSU440[26] by the freeze/thaw method.[27] Hairy root transformation of *M. truncatula* and nodulation studies were performed as described.[17]

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/psb/article/22813

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